The Complement System Facilitates Clearance of Enterococcus faecium during Murine Peritonitis

Masja Leendertse,1,2,3 Rob J. L. Willems,3 Roelof Flierman,5 Alex F. de Vos,1,2 Marc J. M. Bonten,1,4 and Tom van der Poll1,2

1Center for Infection and Immunity Amsterdam and 2Center for Experimental and Molecular Medicine, Academic Medical Center, Amsterdam, 3Department of Medical Microbiology and 4Julius Center for Health Studies and Primary Care, University Medical Center Utrecht, Utrecht, and 5Department of Nephrology, Leiden University Medical Center, Leiden, the Netherlands

Background. Infections with multidrug-resistant enterococci are a growing problem worldwide. Little is known about the host defense against enterococcal diseases. In vitro studies have demonstrated an important role played by complement proteins in neutrophil-mediated phagocytosis. In this study, we investigated the importance of complement in an in vivo model of Enterococcus faecium peritonitis.

Methods. Peripheral neutrophils and peritoneal macrophages were incubated with E. faecium that had been preincubated with decomplemented or normal plasma, and phagocytosis and killing were examined. E. faecium peritonitis was induced in C57BL/6 mice rendered complement deficient by intraperitoneal injection with cobra venom factor (CVF) and in complement 3 (C3) knockout mice. The course of the infection was compared with that in saline control and wild-type mice, respectively, at several time points up to 48 h after infection.

Results. Opsonization by complement enhanced phagocytosis by neutrophils and macrophages. CVF-treated and C3 knockout mice were severely hampered in clearing E. faecium from all organs and tissues under study (peritoneal fluid, blood, lungs, and liver). Higher peritoneal cytokine and chemokine levels were measured in decomplemented mice, whereas no differences in systemic or peritoneal cell kinetics were detected.

Conclusion. Complement deficiency severely hampers the clearance of E. faecium peritonitis and subsequent systemic infection.

Systemic infections with multidrug-resistant enterococci are a major problem in hospitals worldwide. Enterococci have gone from relative triviality to become a leading cause of nosocomial infections and currently rank among the top 3 microorganisms that cause nosocomial bacteremia in the United States [1]. The increase in incidence has been attributed in part to changes in medical care, such as increasing numbers of immunocompromised and critically ill patients, increased use of intravascular devices, more prolonged hospital stays, and widespread use of antibiotics to which the enterococci are resistant [2, 3]. In particular, the ability of Enterococcus faecium to acquire resistance genes has facilitated the increased incidence of enterococcal infection and has led to an increase in the relative importance of E. faecium as a pathogen. At present, resistance to β-lactam and vancomycin has spread almost throughout the whole clinical population of E. faecium; 90% and 80% of all E. faecium involved in nosocomial infections are resistant to ampicillin and to vancomycin, respectively [1].

Surprisingly little is known about either the bacterial virulence factors or the host defense mechanisms that operate in enterococcal infections. After recognition of the microorganism, the immediate host defense consists of the opsonic bactericidal action of complement and phagocytosis by macrophages and/or neutrophils [4]. Elsewhere we investigated the involvement of several components of the innate immune response during E. faecium infection [5–7]. We found that Toll-like receptor 2, signaling via the myeloid differentiation primary-response gene 88 (MyD88) [5], neutrophils [6], and macrophages [7] all make important contributions to the initial host defense against E. faecium infection.
the present study, we investigated the role of the complement system. Previous in vitro studies have demonstrated that complement proteins are of primary importance in neutrophil-mediated killing of *Enterococcus faecalis* and *E. faecium*; therefore, complement proteins were postulated to be essential in mounting an efficient immune response [8, 9]. To our knowledge, however, no studies have subsequently been performed to investigate the role of the complement system in vivo in the host defense against *E. faecium* infections.

In this study, we used a nonlethal mouse model of systemic *E. faecium* peritonitis to investigate the importance of complement in vivo [5]. Cobra venom factor (CVF) was used to deplete mice of complement. In additional experiments, we used C3 gene-deficient mice, which are completely deficient in the late steps of complement activation, regardless of the route used to initiate the complement pathway [10].

**MATERIALS AND METHODS**

**Mice.** Specific pathogen-free 10-week-old female wild-type (WT) C57BL/6 mice were purchased from Harlan Sprague-Dawley (Horst). Age- and sex-matched complement 3 (C3) knockout (KO) mice (which had been backcrossed on a C57BL/6 background for at least 6 generations) were provided by Dr S. Verbeek (Department of Human and Clinical Genetics, Leiden University Medical Center, Leiden, the Netherlands). The Animal Care and Use Committee of the University of Amsterdam approved all experiments.

**Depletion of complement by CVF.** To deplete mice of complement, 4 units of CVF (Quidel) was injected intraperitoneally; control mice were injected with saline. To evaluate the efficiency and additional effects of CVF, 5 mice were injected with either CVF or saline and killed 16 h thereafter, that is, at the moment at which experimental mice were subjected to *E. faecium* peritonitis (see below). For these control mice, C3 levels were measured in plasma and peritoneal fluid, cell counts and differentials were determined, and baseline cytokine levels were measured according to methods described below. From our own experience (M.L. et al, unpublished data, October 2007) and from available literature [11], it was known that mice would have severely reduced levels of complement (<90% of normal values) until at least 3 days after CVF injection.

**Bacterial strain.** Vancomycin-resistant *E. faecium* (strain E155) was used in all experiments. This clinical isolate, from the John H. Stroger, Jr Hospital of Cook County (Chicago, IL), belongs to a genetic subpopulation of hospital-associated *E. faecium* that is responsible for the worldwide emergence of multidrug-resistant *E. faecium*, which is characterized by high-level quinolone and ampicillin resistance, a pathogenicity island that contains the variant *esp* gene, and the presence of 5 cell surface protein genes [12, 13]. For all experiments, the bacteria were grown overnight on sheep blood agar plates and then grown while shaking for ~3.5 h in Todd-Hewitt broth (Difco) at 37°C to the midlogarithmic phase.

**Induction of peritonitis.** Peritonitis was induced as described elsewhere [5]. Mice were injected intraperitoneally with ~10^8 colony-forming units (CFUs) of *E. faecium* in 200 μL of saline. The inoculum was plated immediately after inoculation to determine viable counts. Peritonitis was induced 16 h after injection with either CVF or saline. Additional peritonitis experiments were performed with C3 KO mice.

**Collection of samples.** Mice that were treated with either CVF or saline were killed at 6, 24, or 48 h after infection. C3 KO and WT mice were killed after 6 or 24 h. Mice were anesthetized by inhalation of isoflurane/O2 (2%/2 L; Abbot Laboratories), and a peritoneal lavage was performed with 5 mL of sterile phosphate-buffered saline, using an 18-gauge needle. After collection of peritoneal lavage fluid (PLF), blood was drawn by cardiac puncture, transferred to heparin-gel vacutainer tubes, and immediately placed on ice. Next, the abdomen and thorax were opened, and the liver and lungs were harvested.

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**Figure 1.** Cobra venom factor (CVF) depleting plasma and peritoneal complement. Complement 3 (C3) was measured in plasma (A) and peritoneal lavage fluid (PLF) (B) 16 h after injection with CVF or saline. (In subsequent experiments, mice were infected with *Enterococcus faecium* at this time point). Data are shown as mean ± standard error of the mean of n = 5 mice per group. **P < .01, compared with saline-injected mice.
Figure 2. Diminished phagocytosis of *Enterococcus faecium* preincubated with complement 3 (C3)–depleted plasma. The effect of opsonization by complement on phagocytosis and killing of *E. faecium* was examined. Peripheral blood neutrophils (A, C) and peritoneal macrophages (B, D) harvested from untreated mice were incubated with fluorescein isothiocyanate (FITC)–labeled *E. faecium*, which was preincubated in plasma from mice treated with saline or from mice treated with cobra venom factor (CVF) 16 h earlier (A, B) or harvested from wild-type (WT) or C3 knockout (KO) mice (C, D). Phagocytosis was determined by fluorescein-activated cell sorting analysis and expressed as the mean fluorescence multiplied by the percentage of FITC-positive cells. For killing, peritoneal macrophages (E) were incubated with *E. faecium* that had been preincubated in plasma from saline- or CVF-treated mice (multiplicity of infection, 50), and bacterial killing was assessed over time, shown as the percentage of killed *E. faecium* relative to time *T* = 0. Data are shown as mean ± standard error of the mean of n = 5 mice per group.

**Determination of bacterial outgrowth.** The number of *E. faecium* CFUs in the PLF, blood, liver, and lung homogenate was determined. Organs were weighed and homogenized at 4°C in 4 volumes of sterile saline by use of a tissue homogenizer (Biospect Products). Next, serial 10-fold dilutions were made of each sample in sterile saline, and 50 μL of each dilution was plated onto blood agar plates. Plates were incubated at 37°C under 5% carbon dioxide, and CFUs were counted after 20 h.

**Cell counts and differentials.** Erythrocytes were lysed with ice-cold isotonic ammonium chloride solution (ammonium chloride concentration, 155 mmol/L; potassium bicarbonate concentration, 10 mmol/L; edetic acid concentration, 0.1 mmol/L; pH, 7.4). The remaining cells and the cells in the PLF samples were counted using a Coulter counter (Beckman Coulter). Differential cell counts were performed on cytospin preparations stained with Giemsa (Diff-Quick; Dade Behring). PLF supernatant and plasma were stored at −20°C until determination of the cytokines.

**Opsonophagocytosis and bacterial-killing assay.** Heat-killed (at 70°C for 20 min) bacteria labeled with fluorescein...
isothiocyanate were incubated for 15 min in 20% heparin plasma from saline-treated (16 h earlier), CVF-treated (16 h earlier), or C3 KO mice. To determine the neutrophil phagocytosis capacity, 50 μL of whole blood was incubated with the 2 groups of preincubated bacteria (1 × 10^6 CFUs/mL) for 10, 60, or 120 min at 37°C. Cells were suspended in Quenching solution (Orpegen) and incubated in fluorescein-activated cell sorting (FACS) lysing and fixing solution (Beckton-Dickinson), and then neutrophils were labeled using anti–Gr-1 labeled with phycoerythrin (BD Pharmingen), as recommended by the manufacturer.

Phagocytosis and killing by peritoneal macrophages were evaluated as described elsewhere [5]. Peritoneal macrophages were harvested and resuspended in Roswell Park Memorial Institute 1640 medium at a final concentration of 5 × 10^7 cells/mL and then were allowed to adhere overnight in 48-well microtiter plates (Greiner). For the phagocytosis assay, macrophages were incubated with the preincubated E. faecium (2.5 × 10^7 CFUs/mL) for 10, 60, or 120 min. Phagocytosis was stopped by placing cells on ice; cells were washed in phosphate-buffered saline and suspended in Quenching solution (Orpegen). Neutrophils and macrophages were washed with ice-cold FACS buffer, and then the degree of phagocytosis was determined using the FACSCalibur flow cytometer (Becton Dickinson). For the bacterial-killing assay by peritoneal macrophages, 1 × 10^6 CFUs/mL of E. faecium was first incubated for 15 min in 20% plasma from mice treated with either CVF or saline. The experiment was performed as described elsewhere [5].

Assays. C3 was detected by sandwich enzyme-linked immunosorbent assay (ELISA) as described elsewhere [6]. Levels of macrophage inflammatory protein 2 (MIP-2), cytokine-induced neutrophil chemoattractant (KC), and lipopolysaccharide-induced CXC chemokine (LIX) were measured by ELISA (R&D Systems). Levels of tumor necrosis factor α (TNF-α), interleukin 6 (IL-6), interleukin 10 (IL-10), and monocyte chemoattractant protein 1 (MCP-1) were measured using a commercially available cytometric bead array multiplex assay (BD Biosciences) in accordance with the manufacturer’s recommendations.

Statistical analysis. Data are expressed as mean ± standard error of the mean. Differences between groups were an-
Figure 4. No influence of complement depletion on peritoneal neutrophil and macrophage counts during Enterococcus faecium infection. Sixteen hours after intraperitoneal injection with cobra venom factor (CVF) or saline, mice were inoculated with 10^8 colony-forming units of E. faecium (at time T = 0) and then killed 6, 24, or 48 h thereafter. Peritoneal neutrophils (A) and macrophages (B) are shown. Data are shown as mean ± standard error of the mean of mice per group at each time point. PLF, peritoneal lavage fluid.

Table 1. Effect of Complement Depletion by Cobra Venom Factor (CVF) on Cytokine and Chemokine Responses during Enterococcus faecium Peritonitis

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Mean (SEM) level in plasma, pg/mL</th>
<th>Mean (SEM) level in PLF, pg/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>TFN-α</td>
<td>98 (21)</td>
<td>136 (45)</td>
</tr>
<tr>
<td>IL-6</td>
<td>398 (159)</td>
<td>465 (191)</td>
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<td>IL-10</td>
<td>152 (27)</td>
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<tr>
<td>KC</td>
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<td>26 (3)</td>
</tr>
<tr>
<td>LIX</td>
<td>...</td>
<td>43 (11)</td>
</tr>
<tr>
<td>MIP-2</td>
<td>...</td>
<td>61 (16)</td>
</tr>
</tbody>
</table>

**NOTE.** Mice were inoculated with 10^8 colony-forming units of E. faecium 16 h after CVF or saline injection and killed 6 h after infection. Data are the mean (standard error of the mean [SEM]) of 8 mice per group. IL-6, interleukin 6; IL-10, interleukin 10; KC, cytokine-induced neutrophil chemoattractant; LIX, lipopolysaccharide-induced CXC chemokine; MCP-1, monocyte chemoattractant protein 1; MIP-2, macrophage inflammatory protein 2; PLF, peritoneal lavage fluid; TNF-α, tumor necrosis factor α.

Results

**RESULTS**

Complement improves phagocytosis of E. faecium by neutrophils and macrophages but does not influence intracellular killing. E. faecium was preincubated in plasma from saline-treated mice or from CVF-treated mice. CVF treatment led to a systemic and peritoneal reduction of the C3 protein of >90% (Figure 1). E. faecium that was preincubated in normal plasma from saline-treated mice was phagocytosed more rapidly by neutrophils and by macrophages than was E. faecium incubated in plasma depleted of complement by CVF (P < .001) (Figure 2A and 2B). The selective absence of C3 in plasma (from blood obtained from C3 KO mice) also impaired phagocytosis of E. faecium by neutrophils and macrophages, albeit to a lesser extent than was observed for plasma from CVF-treated mice (Figure 2C and 2D). Once internalized, opsonization by complement did not influence killing by macrophages (Figure 2E).

Impaired clearance of E. faecium in complement-depleted mice. CVF-treated mice remained depleted of C3 during the entire experiment (data not shown). Sixteen hours after treatment with either CVF or saline, the mice were subjected to E. faecium peritonitis and killed 6, 24, or 48 h thereafter. Complement depletion caused a severe delay in E. faecium clearance (Figure 3). Six hours after the start of infection, >2-log higher numbers of E. faecium CFUs were cultured from PLF of CVF-treated mice compared with PLF from saline-treated control mice (Figure 3A). Six hours after the start of infection, 1 log more E. faecium CFUs were cultured from the blood (Figure 3B), liver (Figure 3C), and lungs (Figure 3D) of CVF-treated mice compared with those from saline-treated mice. Both CVF- and saline-treated mice were able to reduce peritoneal and systemic E. faecium burdens. At 24 and 48 h after infection, peritoneal E. faecium CFU numbers were comparable in both groups of mice, whereas the amount of E. faecium in the blood, liver, and lungs of CVF-treated mice remained elevated compared with the amount in those of control mice.

Effect of complement depletion on the inflammatory response. At the time of infection, leukocyte counts and differentials in PLF and in blood from CVF-treated mice and from saline-treated mice were similar (Figure 4 and data not shown). Six hours after induction of E. faecium peritonitis, CVF- and
Complement in Enterococcus faecium Peritonitis

Complement 3 (C3) knockout (KO) mice are impaired in peritoneal and systemic Enterococcus faecium clearance. C3 KO and wild-type (WT) mice were intraperitoneally infected with 10⁵ colony-forming units (CFUs) of E. faecium and killed 6 or 24 h thereafter. E. faecium outgrowth in peritoneal fluid (A), blood (B), liver (C), and lung homogenate (D) are shown. Data are shown as mean ± standard error of the mean of or 8 mice per group at each time point. *P < .05, **P < .01, compared with WT mice.

Figure 5. Complement 3 (C3) knockout (KO) mice are impaired in peritoneal and systemic Enterococcus faecium clearance. C3 KO and wild-type (WT) mice were intraperitoneally infected with 10⁵ colony-forming units (CFUs) of E. faecium and killed 6 or 24 h thereafter. E. faecium outgrowth in peritoneal fluid (A), blood (B), liver (C), and lung homogenate (D) are shown. Data are shown as mean ± standard error of the mean of or 8 mice per group at each time point. *P < .05, **P < .01, compared with WT mice.

saline-treated mice had attracted similarly high amounts of neutrophils into the peritoneal cavity (Figure 4A). As the amount of peritoneal E. faecium decreased, neutrophil numbers in the PLF decreased, returning to near baseline values after 48 h in both groups (Figure 4A). E. faecium peritonitis resulted in an initial decrease in the number of peritoneal macrophages, as measured 6 h after infection (Figure 4B), confirming our previous results [7]. Thereafter, macrophage numbers increased again, showing modestly higher counts from 24 h onward. Although after 24 h the CVF-treated mice tended to have lower numbers of macrophages in the PLF than did saline-treated mice, the difference was not statistically significant. During infection, no differences were found between the groups with regard to circulating neutrophils and monocytes (data not shown).

To evaluate further the inflammatory response in complement-deficient mice and in control mice, we measured levels of the proinflammatory cytokines TNF-α and IL-6 and the anti-inflammatory cytokine IL-10, as well as levels of MCP-1, in PLF and in blood and the neutrophil-attracting CXC chemokines LIX, KC, and MIP-2 in PLF. At the time of infection, both CVF- and saline-treated mice had low or undetectable cytokine and chemokine levels that did not differ between the 2 groups (data not shown). CVF-treated mice killed 6 h after infection showed higher peritoneal TNF-α and IL-6 levels than did saline-treated control mice, with correspondingly 2-log higher numbers of CFUs of E. faecium in the peritoneal cavity (Table 1). Peritoneal IL-10 and MCP-1 levels were comparable in both groups, as were plasma cytokine levels. The concentrations of KC, MIP-2, and LIX were all higher in CVF-treated mice. Strong positive correlations were found between peritoneal bacterial loads and levels of the chemokines KC (r = 0.78; P < .001), LIX (r = 0.72; P < .005), and MIP-2 (r = 0.69; P < .005). Cytokine levels measured at later time points were undetectable or low in both CVF-treated mice and saline-treated mice (data not shown).

Impaired clearance of E. faecium in C3 KO mice. CVF treatment did not render mice absolutely depleted of complement (Figure 1) and may have had other (toxic) effects. Therefore, we wished to confirm the data reported above by using
C3 KO mice had higher numbers of *E. faecium* chemokines KC (\( \beta \)), LIX (\( \beta \)), and were found between peritoneal bacterial loads and levels of the C3 KO mice 6 h after infection (Table 2). Positive correlations to KC, LIX, and MCP-1 chemokine levels were measured in mice, either (data not shown).

C3 KO mice and C3 KO mice. During infection, no differences were found in peritoneal and peripheral cell composition or cytokine profiles between uninfected WT liver. No differences were found in peritoneal and peripheral cell composition or cytokine profiles between uninfected WT mice (Figure 5A). In the blood, liver, and lungs, \(~2\) log more *E. faecium* CFUs were cultured from C3 KO mice than from WT mice (Figure 5B–5D). Twenty-four hours after infection, the C3 KO mice had higher numbers of *E. faecium* CFUs in the PLF, blood, and lungs compared with those of WT mice, yet the C3 KO mice had been able to reduce the amount of bacteria, even to an extent similar to that observed for WT mice in the liver. No differences were found in peritoneal and peripheral cell composition or cytokine profiles between uninfected WT mice and C3 KO mice. During infection, no differences were found in these cell compositions between WT mice and C3 KO mice, either (data not shown).

Higher peritoneal and blood IL-6 levels and peritoneal MIP-2, KC, LIX, and MCP-1 chemokine levels were measured in C3 KO mice 6 h after infection (Table 2). Positive correlations were found between peritoneal bacterial loads and levels of the chemokines KC \( (r = 0.57; P < .05) \), LIX \( (r = 0.64; P < .01) \), and MIP-2 \( (r = 0.53; P < .05) \). Levels of TNF-\( \alpha \) and IL-10 were not found to differ in either plasma or PLF. No differences were measured after 24 h.

**DISCUSSION**

To our knowledge, this study demonstrates, for the first time, the involvement of the complement system in the host defense against *E. faecium* infection in vivo. *E. faecium* has emerged as an important pathogen that causes severe nosocomial infections worldwide. Because of the growing resistance of *E. faecium* to almost all antibiotics used in the hospital setting, infections with this pathogen are difficult to treat. To improve therapeutic options, a better understanding of the pathogenesis of these infections and of the interaction with the host defense systems is needed. Previous in vitro studies indicated an important role for complement proteins in opsonizing enterococci and in subsequent phagocytosis by neutrophils [8, 9]. After confirming that opsonization by complement enhances neutrophil and, additionally, macrophage phagocytosis of our clinical *E. faecium* isolate, we investigated the role of the complement system during *E. faecium* infection in vivo. Both groups of complement-deficient mice (mice decomplemented by use of CVF injection and C3 KO mice) were severely hampered in clearing *E. faecium* peritonitis. Early in the infection, complement-deficient mice had \(~2–3\) log higher numbers of *E. faecium* CFUs cultured from the peritoneal fluid, resulting in higher systemic dissemination and a severe delay in *E. faecium* clearance. Higher *E. faecium* burden was accompanied by higher peritoneal cytokine and chemokine levels and higher systemic IL-6 levels in C3 KO mice.

Interestingly, complement deficiency did not hamper cell recruitment to the primary site of infection during *E. faecium* peritonitis, although we cannot exclude the possibility that very early after infection (ie, within the first few hours) complement deficiency does have an effect on cell influx into the peritoneal cavity.

The complement system is a key player in the systemic and peritoneal defense system [14]; it comprises one of the major groups of pattern-recognition molecules, consisting of \( >30 \) serum and cell-surface proteins. Activation of complement can occur through the classical, alternative, or mannos-binding lectin pathways, all of which rely on the function of complement component C3. Cleavage of C3 by the C4b2a (classical and lectin pathways) or C3bBb (alternative pathway) complexes is a critical step that results in the release of the soluble anaphylatoxin C3a and the deposition of C3b and iC3b on the surface of the bacterium. C3b and iC3b serve as ligands for cellular receptors on leukocytes, and binding leads to microbial clearance by phagocytes. In addition, cleavage of C3 results in the formation of the membrane attack complex (C5–C9). In the complete absence of C3, almost all of the biological properties mediated by complement are absent, including opsonization and phagocytosis of bacteria, directed migration of inflammatory cells, and amplification of the immune response [15–17]. In general, the thick cell wall of gram-positive bacteria (such as *E. faecium*) prevents complement-mediated lysis; therefore, complement primarily plays an opsonic role in the host defense against gram-positive bacterial infections [18, 19]. Interestingly, Nordahl et al [20] demonstrated a direct anti-

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**Table 2. Plasma and Peritoneal Cytokines in Wild-Type (WT) and Complement 3 (C3) Knockout (KO) Mice Intraperitoneally Infected with** \( 10^8 \) **Colony-Forming Units of Enterococcus faecium**

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Mean (SEM) level in plasma, pg/mL</th>
<th>Mean (SEM) level in PLF, pg/mL</th>
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<tbody>
<tr>
<td>TNF-( \alpha )</td>
<td>65 (11)</td>
<td>99 (17)</td>
</tr>
<tr>
<td>IL-6</td>
<td>317 (53)</td>
<td>2077 (1058)*</td>
</tr>
<tr>
<td>IL-10</td>
<td>118 (29)</td>
<td>156 (37)</td>
</tr>
<tr>
<td>MCP-1</td>
<td>416 (90)</td>
<td>605 (199)</td>
</tr>
<tr>
<td>KC</td>
<td>...</td>
<td>44 (7)</td>
</tr>
<tr>
<td>LIX</td>
<td>...</td>
<td>32 (6)</td>
</tr>
<tr>
<td>MIP-2</td>
<td>...</td>
<td>52 (9)</td>
</tr>
</tbody>
</table>

**NOTE.** C3 KO and WT mice were inoculated with \( 10^8 \) colony-forming units of *E. faecium* and killed 6 h thereafter. Data are the mean (standard error of the mean [SEM]) of 8 mice per group. IL-6, interleukin 6; IL-10, interleukin 10; KC, cytokine-induced neutrophil chemoattractant; LIX, lipopolysaccharide-induced CXC chemokine; MCP-1, monocyte chemoattractant protein 1; MIP-2, macrophage inflammatory protein 2; PLF, peritoneal lavage fluid; TNF-\( \alpha \), tumor necrosis factor \( \alpha \).

\* \( P < .05 \) compared with WT mice.
microbial effect of C3a and C3a-desArg, which efficiently killed *E. faecalis*.

Previous in vitro studies demonstrated that neutrophil-mediated bactericidal activity toward enterococci was ablated by inactivation of complement. Complement 4–deficient serum consistently promoted neutrophil killing, which suggests that complement-mediated neutrophil activity may proceed by the alternative pathway [8, 9]. Additional studies demonstrated enhanced killing of enterococci when specific antibodies were involved. Huebner et al [21] found that enterococcal glucose-glycerol-teichoic acid capsular polysaccharides are targeted by antibodies in rabbits, and that antienterococcal rabbit serum had a protective effect in mice infected with enterococci. In an opsonophagocytic-killing assay, antiserum raised against purified polysaccharide from *E. faecalis* killed a variety of heterologous strains of *E. faecalis* as well as *E. faecium*. Interestingly, certain strains of *E. faecium* were found to be resistant to phagocytosis by neutrophils, probably because of a carbohydrate-containing moiety expressed by these strains. Specific antibodies could overcome this resistance, most likely by promoting deposition of complement [22–24]. In addition, extracellular gelatinase expressed by *E. faecalis* destroys complement in human serum, by hydrolyzing C3a and degenerating C3b [25, 26]. Expression of extracellular gelatinase was also described for *E. faecium* elsewhere [27]; this, along with the expression of capsular polysaccharides described above, may also contribute to the resistance of *E. faecium* to phagocytosis.

The clinical isolate of *E. faecium* (strain E155) used in our experiments belongs to a genetic subpopulation of hospital-associated *E. faecium* that is responsible for the worldwide emergence of nosocomial multidrug-resistant *E. faecium* infections [12, 13]. We first established, by means of an in vitro assay, that opsonization by complement of this *E. faecium* strain enhanced phagocytosis by neutrophils in whole blood and by primary peritoneal macrophages. This is important because peritoneal macrophages are the cells that are first encountered by the bacteria when they are introduced into the normally sterile peritoneal cavity [14]. This strain was clearly not resistant to phagocytosis.

We then tested the influence of complement in vivo. CVF forms a C3 convertase that rapidly and uncontrollably cleaves C3, resulting in hyperactivation of the complement cascade, thereby degrading the complement components and thus resulting in nearly complete depletion of complement activity [11, 28]. After injection with CVF, mice will remain decomplemented for up to 3 days [11]. Decomplementation by CVF resulted in 90% reduction of C3 levels. CVF-treated mice were severely debilitated in clearing enterococci, which was especially apparent early after infection. Six hours after the start of infection, CVF-treated mice had up to 3 log more *E. faecium* CFUs in the peritoneal cavity and increased systemic dissemination, compared with control mice. Despite this prolonged increased bacterial load, no mortality was observed, and apparently bacteria were not able to multiply in vivo, because at later time points the bacterial numbers were also reduced in decomplemented mice. Although CVF treatment caused a severe reduction in systemic complement proteins, some C3 could still be measured; it could potentially still have played a role in the reduction of the bacteria. We then used C3 KO mice to examine whether an absolute deficiency in C3 would render the host even more hampered in enterococcal clearance. Like the experiments with CVF-treated mice, these experiments resulted in higher bacterial loads 6 h after infection in the C3 KO mice than in the WT control mice, as well as a reduction in load later in the infection.

Both cleavage products C3a and C5a are known anaphylatoxins that play a role in attracting leukocytes to the site of inflammation [29–31]. Interestingly, we did not find differences in the attraction of neutrophils or macrophages into the peritoneal cavity in either model of complement-deficient mice. As such, the increased numbers of *E. faecium* CFUs in the peritoneal cavity were not due to reduced peritoneal cellular recruitment. Apparently, in the absence of an intact complement system, multiple compensatory mechanisms exist for the recruitment of neutrophils into the PLF after *E. faecium* infection. In this respect, it should be noted that the peritoneal levels of the neutrophil-attracting CXC chemokines KC, MIP-2, and LIX were elevated in complement-deficient mice 6 h after infection, which was strongly correlated with the higher bacterial loads; these elevated local CXC chemokine concentrations possibly compensated for the absent complement-attracting function. Our data show that complement is required for the early containment of *E. faecium* infection and that the absence of complement causes increased dissemination and severely hampered clearance of the bacteria, which strongly suggests that complement is pivotal in the early antibacterial response during *E. faecium* infection, primarily because it facilitates phagocytosis.

Acknowledgments

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References


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